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13. ABSTRACT *(Maximum 200 Words)*

Increased awareness and systematic screening for breast cancer has resulted in early detection of this disease. However, 20 – 30% of node negative breast carcinoma patients will develop recurrent tumors and given the highly metastatic nature of breast cancer, many of these patients progress to disseminated disease. Although developments in experimental therapies for the treatment of advanced breast cancer is promising, much of the existing treatment for advanced metastatic cancer is palliative. Among the numerous **symptoms of advanced cancer**, pain remains the most significant determinant of **quality of life**. Despite pain being the most feared symptom of advanced cancer, clinical management of cancer pain remains inadequate. Limitation in the clinical management of advanced cancer pain appears multifactorial ranging from the nature of pain itself to the irrational fear of prescribing large quantities of opiates among the treating physicians . The goal of this investigation is to develop a novel non-opioid approach to pain management. Our present focus is on antisense-oligonucleotide-mediated knock down of an intracellular signal transduction molecule protein kinase C-γ known to play a critical role in the development of pathological pain. If successful, a new therapeutic modality will have wide spread applications in the management of terminal cancer pain and other intractable pain syndromes.

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Table of Contents

4. Introduction

Increased awareness and systematic screening for breast cancer has resulted in early detection of this disease. However, 20 – 30% of node negative breast carcinoma patients will develop recurrent tumors [Weidner, 1995] and given the highly metastatic nature of breast cancer, many of these patients progress to disseminated disease [Lee, 1983]. Although developments in experimental therapies for the treatment of advanced breast cancer is promising, much of the existing treatment for advanced metastatic cancer is palliative. Among the numerous **symptoms of advanced cancer**, pain remains the most significant determinant of **quality of life** [Heim & Oci, 1993; Portenoy, 1990; Daut & Cleeland, 1982]. Despite pain being the most feared symptom of advanced cancer, clinical management of cancer pain remains inadequate. Limitation in the clinical management of advanced cancer pain appears multifactorial ranging from the nature of pain itself to the irrational fear of prescribing large quantities of opiates among the treating physicians [Fife et al, 1993; Zenz & Strumpf, 1993]. A novel non-opioid approach to pain management which requires minimal high-technology resources will have wide spread applications in the management of terminal cancer pain and other intractable pain syndromes.

In this proposal, we describe a **novel viral-vector mediated gene-therapeutic approach to pain management**. This notion is based on well documented observations that all vertebrate nervous system including humans posses an endogenous analgesic system. Of the numerous neurotransmitter systems implicated in such an endogenous analgesic system, one of the best described descending analgesic mechanism is the brain stem serotonergic input to the spinal cord. Stimulation of brain stem neurons result in release of serotonin, an amine neurotransmitter, at the spinal cord which modulates the transmission along the pain pathway. The original goal of this project was to examine if enhancement of this endogenous serotonergic analgesic system can provide analgesia. Our approach was to introduce a recombinant adenovirus designed to express one subtype (5HT3) of the serotonin receptor into the subarachnoid space as a means of overexpressing the 5HT3 receptor in spinal neurons. As documented on the last progress report, we concluded that intrathecal Ad(5HT3-sense) administration had little effect on nociceptive threshold in rats. A reexamination of adenoviral access to the spinal cord proper led us to conclude that a 100 nm adenovirus particle was too large to cross into the spinal cord proper even after subarchnoid administration. This led us to propose an alternative approach to gene therapy for pain managment through intrathecal injection of antisense oligonucleotide targeting protein kinase C-γ, an intracellular signal transduction molecule well documented to play a critical role in neuropathic pain.

5. Body

In this reporting period (Year II of the project), we focused our efforts on the development of an alternative strategy for gene-based therapy for neuropathic pain. Specifically, we targeted an intracellular pronociceptive signal transduction molecule, protein kinase C-γ, strongly implicated in mediating the pain cascade [Mao et al, 1995; Malmberg et al, 1997], and began investigating the use of antisense oligonucleotide [Agrawal & Temsamani, 1996; Akhtar & Agrawal, 1997] as a potential therapeutic drug. The antisense oligonucleotide approach was chosen because the PI believes that this strategy, in addition to its proven utility as a selective

drug in other systems, is a technology most likely to lead into a human clinical trial in the near future [Akhtar & Agrawal, 1997; Diasio & Zhang, 1997].

The following progress have been made on the revised Task II stated on the last progress report:

Task II:

- *perform further in vitro antisense oligonucleotide PKC-γ knock down experiments to verify the mechanism of protein knock down.*

1. Several candidate antisense oligonucleotides were identified based on sequence comparison of the classical PKCs (figure 1). *In vitro* antisense knock down experiment demonstrates differential efficacy in reducing the amount of PKCγ protein (figure 2). All 11 oligonucleotides tested to date (O1 – O11) demonstrate some degree of reduction in PKC-γ protein. O4, O6, and O9 demonstrating best knock down. The result for O6 is in agreement with our preliminary data presented on our last progress report. In contrast, O7 show only a modest efficacy.
2. We have encountered problems in demonstrating efficient antisense knock down of PKC-γ, in vivo. Various dosing regimen have been tried (up to 100 μg / dose x q 12 hours) without consistent correlation between behavioral analgesia and biochemical evidence of spinal cord PKC-γ protein reduction. We have revised our behavioral pain assay from a partial sciatic nerve ligation to a formalin (inflammatory) model of pain. Since chronic pain associated with cancer often exhibits a strong inflammatory component, the formalin model may be more appropriate.

During the next funding period, the following aims will be accomplished:

a. A time course experiment to develop an optimal protocol for in vivo knock down using Western blot assay of spinal cord homogenate.
b. A time course study to examine the return of PKC-γ protein level after cessation of the antisense treatment. This information will help design an *in vivo* protocol to examine whether interruption of the pain cascade will provide a long-term relief from pain. Alternatively, pain could return immediately after cessation of the antisense treatment as the cells regenerate PKC-γ.
c. *In vivo* studies examining anti-nociceptive effects of anti-PKC-γ antisense oligonucleotide will be continued with focus on the formalin model of inflammatory pain.

6. Key Research Accomplishments:

- Developed an alternative strategy based on antisense oligonucleotide knock down of protein kinase C-γ playing a critical role in the neuropathic cascade.
- Screened antisense oligonucleotides to identify a sequence leading to selective PKC-γ protein knock down.
- Demonstrated anti-nociceptive effect of intrathecal administration of antisense oligonucleotide targeting PKC-γ.

7. Reportable Outcomes:

Manuscripts / abstracts / presentations:
Wu C, Garry M, Zollo R, Yang J "Gene therapy for the management of pain Part I: Methods and strategies", Anesthesiology, (in revision).

Wu C, Garry M, Zollo R, Yang J "Gene therapy for the management of pain Part II: Molecular targets", Anesthesiology, (in revision).

Zollo R, Malik S, Yu J, Yang J "Antisense oligonucleotide-mediated selective knock down of protein kinase C γ", (in preparation).

Yang J, Garry M, Zollo RA "Protein kinase C-gamma and neuropathic pain", Abstract FF-11, Era of Hope Meeting, 6/8-12,2000, Atlanta, GA.

8. Conclusions:

Inadequate management of cancer pain has been widely documented and may effect a reduction of quality of life in terminal patients [Heim & Oci, 1993; Portenoy, 1990; Daut & Cleeland, 1982]. Furthermore, neuropathic pain, which may be present with advanced cancer is generally resistant to opioid therapy [Payne, 1993; Arner & Myerson, 1988]. Development of a non-opiate, non-addictive, long-lasting therapy for cancer pain will revolutionarize clinical cancer pain management and alleviate patient suffering. The goal of this proposal is to investigate the feasibility of a gene-therapeutic approach to pain management. Should this approach prove feasible, in future work, this concept can be extended to overexpression of other neurotransmitter receptors implicated in the antinociceptive action (e.g. $GABA_A$, adrenergic α2, etc.) or underexpression of receptors mediating the nociceptive actions (e.g. NMDA, tachykinin, etc.) through antisense knock down.

The original goal was to use the recombinant adenovirus as a vehicle for overexpressing antinociceptive serotonin type 3A receptors in the spinal cord as a means of providing pain relief. Our results over the first 12 months indicated that the recombinant adenovirus delivered by the subarachnoid route will not work. The physical barrier probably due to the marginal glial cells of the spinal cord prevents the adenovirus from transducing spinal cord neurons. Without efficient transduction of the spinal cord proper, the proposed approach will not work. Direct injection of the virus into the spinal cord is an alternative delivery method, but remains unsatisfactory because the approach is too invasive for easy translation to the human clinical arena.

We have taken an alternative strategy to accomplish the same goal of developing a treatment for neuropathic pain. During the present reporting period, we explored antisense oligonucleotide targeting spinal cord protein kinase C-γ as a therapeutic strategy for a non-opioid pain management. Progress thus far has identified several candidate antisense oligonucleotides that exhibit PKC-γ protein knock down. We will continue to this investigate antisense oligonucleotide as a novel alternative to opioids for treating neuropathic pain. Antisense oligonucleotide is now well accepted as a form of therapy in humans. It is our goal to thoroughly investigate the antisense oligonucleotide

targeting spinal cord PKC-γ in a preclinical model and move towards implementing a clinical trial in humans. Task for year 3 of funding will focus on in vivo assay of efficacy of PKC-γ antisense oligonucleotide. We hope to move a step closer to the goal of developing a novel and effective pain relief with reduced side-effects for combating the diverse types of pain associated with advanced cancer. In this respect, the antisense oligonucleotide approach may be more practical than the viral vector approach.

9. References:

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Heim, H.M. and Oci, T.P.S. (1993) *Comparison of prostatic cancer patients with and without pain.* Pain **53:** 159 - 162.

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Malmberg A, Chen C, Tonegawa S, Basbaum AI (1997) *Preserved acute pain and reduced neuropathic pain in mice lacking PKC-γ,* Science **278**: 279 – 283.

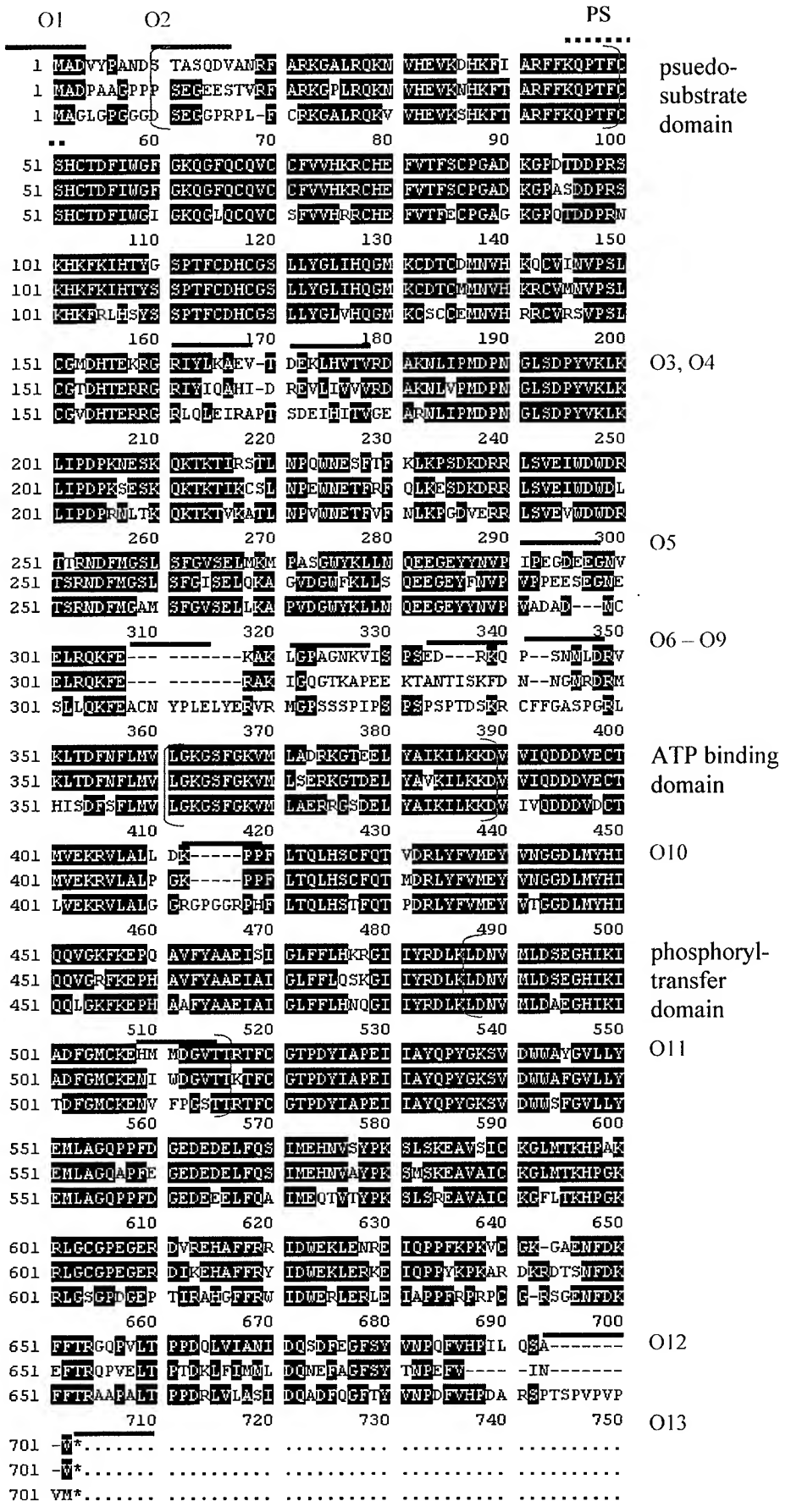
Mao J, Price DD, Phillips LL, Lu J, Mayer DJ (1995a) *Increases in protein kinase C gamma immunoreactivity in the spinal cord dorsal horn of rats with painful neuropathy*, Neuroscience Letter **198**: 75 – 78.

Pourtenoy, R.K. (1990) *Pain and quality of life: clinical issues and implications for research.* Oncology **4**: 172 - 180.

Weidner N (1995) *Prognostic factors in breast carcinoma*, Current Opin. Ob & Gyn 7: 4 – 9.

Zenz, M. and Willweber-Strumpf, A. (1993) *Opiophobia and cancer pain in Europe.* Lancet, **341**: 1075-6.

Figure 1: *Amino acid sequence alignment of classical rat PKC isoenzymes.* The entire coding sequence for PKC-α (E04372), PKC-β1 (M19007), and PKC-γ (E04371) were aligned using DNAsis (Hitachi Inc, CA). Genebank accession numbers are given in parenthesis. Regions of significant amino acid divergence were identified and thirteen oligonucleotides corresponding to the selected amino acid regions were defined as the candidate antisense oligonucleotides noted as O1 – O13 (noted in red). The PS oligonucleotide (in blue) was designed against a conserved region in the psuedosubstrate domain. Three of the well defined functional domains of the classical PKCs are identified by the green brackets. PKC-βII with 95% amino acid identity with βI was omitted from the figure for clarity.



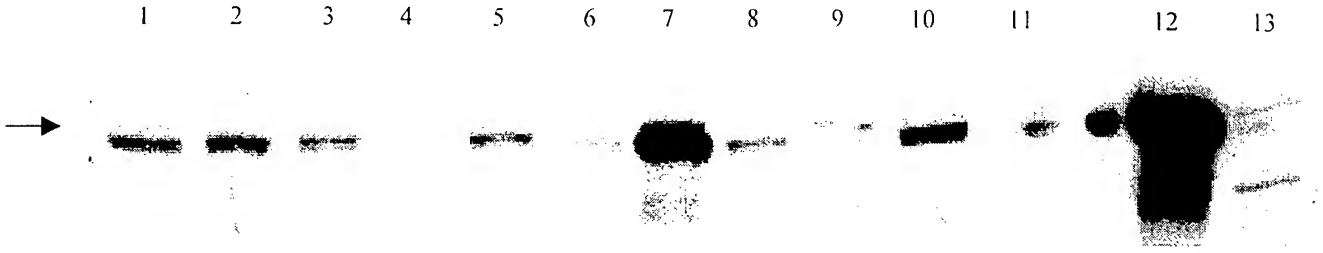


Figure 2: *Differential reduction of PKC-γ by selected antisense oligonucleotides.* Western blot of PKC-γ protein in antisense oligonucleotide-treated cells. Lanes 1 – 11 corresponds to antisense oligonucleotides O1 – O11 shown in figure 1. Lane 12 is a postive control without oligonucleotide treatment and lane 13 is a negative control. Individual 35 mm tissue culture dishes 70% confluent with HEK293 cells were co-transfected with PKCγ-pCIneo (0.2 μg / dish) and 1.0 μg / dish oligonucleotide using Lipofectamine Plus (Gibco). Twenty four hours after termination of transfection, cells were harvested and equal amount of protein loaded and separated by SDS gel electrophoresis.